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1. Your reference
   G21715P

2. Patent application number
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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*
   CHIRON S.p.a.
   Via Fiorentina, 1
   53100 Siena
   ITALY

   Patents ADP number *(if you know it)*
   71578100 1

   If the applicant is a corporate body, give the country/state of its incorporation
   ITALY

4. Title of the invention
   Hepatitis C Receptor Protein

5. Name of your agent *(if you have one)*
   CARPMAELS & RANSFORD

   "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*
   43, Bloomsbury Square
   London WC1A 2RA

   Patents ADP number *(if you know it)*
   83001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
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Patents Form 1/77

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Continuation sheets of this form	
Description	12
Claim(s)	2
Abstract	1
Drawing(s)	3

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
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11. I/We request the grant of a patent on the basis of this application.

Signature Cepmael Hallybone Date 6/10/97

12. Name and daytime telephone number of person to contact in the United Kingdom

HUW G. HALLYBONE

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Field of the Invention

The present invention relates to the use of CD81 protein and nucleic acid in the therapy and diagnosis of hepatitis C and to pharmaceutical compositions, animal models and diagnostic kits for such uses.

Brief Description of the Prior Art

HCV (previously known as Non-A Non-B hepatitis - NANBV) is a positive sense RNA virus of about 10000 nucleotides with a single open reading frame encoding a polyprotein of about 3000 amino acids. Although the structure of the virus has been elucidated by recombinant DNA techniques (European patent application EP-A-0318216 and European patent application EP-A-0388232), the virus itself has not been isolated and the functions of the various viral proteins produced by proteolysis of the polyprotein have only been inferred by analogy with other similar viruses of similar genomic organisation (Choo *et al* PNAS USA (1991) 88 2451-2455).

The viral proteins are all available in recombinant form, expressed in a variety of cells and cell types, including yeast, bacteria, insect and mammalian cells (Chien, D.Y. *et al* PNAS USA (1992) 89 10011-10015 and Spaete, R.R. *et al* Virology (1992) 188 819-830).

Two proteins, named E1 and E2 (corresponding to amino acids 192-383 and 384-750 of the polyprotein respectively) have been suggested to be external proteins of the viral envelope which are responsible for the binding of virus to target cells.

HCV research is hindered very considerably by the limited host range of the virus. The only reliable animal model for HCV infection is the chimpanzee and it is not possible to propagate HCV in tissue culture.

In our copending International patent application PCT/IB95/00692 (WO 96/05513), we describe a method employing flow cytometry to identify cells carrying the HCV receptor. We have shown that, by labelling cells with recombinant E2 envelope protein, it is possible to sort cells using flow cytometry, isolating those cells capable of specific binding to the E2 and therefore potentially carrying the HCV receptor.

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In our copending International patent application PCT/IB96/00943 (WO 97/09349), we have identified a protein capable of binding to the E2 envelope protein of HCV.

We have now succeeded with some difficulty in cloning the DNA encoding the HCV receptor and have discovered, surprisingly that the DNA encodes a cellular protein known as CD81. We are not aware of any association in the literature between CD81 and the HCV. CD81 was first identified by monoclonal antibodies as the target of an antiproliferative antibody (TAPA-1) which inhibited *in vitro* cellular proliferation. Armed with this new information and given the sequence knowledge of CD81 in the public databases it is now possible to design and produce an armoury of therapeutic and diagnostic reagents against HCV.

Summary of the Invention

According to the present invention, there is provided a CD81 protein, or a functional equivalent thereof for use in the therapy or diagnosis of hepatitis C (HCV).

The term "CD81 protein, or a functional equivalent thereof" as used herein means the CD81 protein as defined by the protein sequence listed in the SWISSPROT database (Accession No. P18582) or the EMBL/GENBANK database (Accession No. M33690) or a functional equivalent thereof.

A functional equivalent of CD81 is a protein which is capable of binding to HCV, preferably to the E2 protein of HCV. The functional equivalentmay be an analogue of CD81 or a fragment thereof.

The term "a functionally equivalent fragment" as used herein means any fragment or assembly of fragments of the complete protein. For example, the complete protein may be truncated at one or both ends or domains may be removed provided the protein retains the defined function. For example, one or more regions of the protein responsible for membrane binding may be removed to render the protein soluble when produced by a recombinant process.

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The term "a functionally equivalent analogue" as used herein means any analogue of CD81 obtained by altering the amino acid sequence, for example by one or more amino acid deletions, substitutions or additions such that the protein analogue retains the ability to bind to HCV, preferably the E2 protein of HCV. Amino acid substitutions may be made, for example, by point mutation of the DNA encoding the amino acid sequence.

The functional equivalent of CD81 may be an analogue of a fragment of CD81. The CD81 or functional equivalent may be chemically modified provided it retains its ability tobind to HCV, preferably the E2 protein of HCV.

The CD81 protein, or functional equivalent thereof may be produced by recombinant DNA expression or by chemical synthesis.

In order to produce sufficient amounts of CD81 protein, or functional equivalent thereof for use in accordance with the present invention, expression may conveniently be achieved by culturing under appropriate conditions recombinant host cells containing the CD81 protein, or functional equivalent thereof.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, mammalian cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells and many others. A common, preferred bacterial host is *E. coli*.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*,

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Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

According to a further aspect of the invention, there is provided a method for treating an infection of HCV comprising administering to a patient a therapeutically effective amount of CD81 protein, or afunctional equivalent thereof effective to reduce the infectivity of the virus.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Since the infection mechanism of HCV appears to depend, in part, upon the availability of a cell surface receptor, making available a soluble form of the CD81 protein, or a functional equivalent thereof will act as an antagonist of binding of HCV to the cellular receptor thus reducing or preventing the infection process and thereby treating the disease.

A suitable soluble form of the CD81 protein, or a functional equivalent thereof might comprise, for example, a truncated form of the protein from which the transmembrane domain or domains have been removed either be a protein cleavage step or, by design, in a chemical or recombinant DNA synthesis.

Alternatively, a hybrid particle comprising at least one particle-forming protein, such as hepatitis B surface antigen or a particle-forming fragment thereof, in combination with the CD81 protein or functional equivalent thereof could be used as an antagonist of binding of HCV to the cellular receptor.

According to a further aspect of the invention, there is provided a pharmaceutical composition comprising a CD81 protein or functional equivalent thereof, optionally as a pharmaceutically acceptable salt, in combination with a pharmaceutically acceptable carrier.

The pharmaceutical composition may be in any appropriate form for administration including oral and parenteral compositions.

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A process is also provided for making the pharmaceutical composition, in which a protein of the present inventionis brought into association with a pharmaceutically acceptable carrier.

According to a further aspect of the invention, there is provided a CD81 protein or functional equivalent thereoffor use as a pharmaceutical.

According to a further aspect of the invention, there is provided the use of a CD81 protein or functional equivalent thereofin the manufacture of a medicament for the treatment of an HCV infection.

The ability of a CD81 protein or functional equivalent thereofto bind to HCV permits the use of the proteinas a diagnostic for HCV infection, for example in an ELISA or RIA.

A soluble form of the protein could, for example, be used in an ELISA form of assay to measure neutralising antibodies in serum.

According to a further aspect of the invention, there is provided an assay for HCV antibodies in a serum sample comprising the step of allowing competitive binding between antibodies in the sample and a known amount of an HCV protein for binding to a CD81 protein or functional equivalent thereofand measuring the amount of the known HCV protein bound.

Preferably, the CD81 protein or functional equivalent thereof is immobilised on a solid support and the HCV protein, which may suitably be E2 HCV envelope protein, optionally recombinant E2 protein, is labelled, preferably enzyme labelled.

In an assay of this form, competitive binding between antibodies and the HCV protein for binding to the CD81 protein or functional equivalent thereof results in the bound HCV protein being a measure of antibodies in the serum sample, most particularly, HCV neutralising antibodies in the serum sample.

A significant advantage of the assay is that measurement is made of neutralising antibodies

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directly (i.e those which interfere with binding of HCV envelope protein to the cellular receptor). Such an assay, particularly in the form of an ELISA test has considerable applications in the clinical environment and in routine blood screening.

Also, since the assay measures neutralising antibody titre, the assay forms a ready measure of putative vaccine efficacy, neutralising antibody titre being correlated with host protection.

In a further aspect of the invention, there is provided a diagnostic kit comprising the CD81 protein or functional equivalent thereof. Preferably the kit also contains at least one HCV labelled HCV protein, optionally enzyme labelled.

The CD81 protein or functional equivalent thereofmay be used to screen for chemical compounds mimicking the HCV surface structure responsible for binding to the HCV receptor.

According to a further aspect of the invention, there is provided a method for screening chemical compounds for ability to bind to the region of HCV responsible for binding to a host cell, comprising measuring the binding of a chemical compound to be screened to a CD81 protein or functional equivalent thereof.

This aspect of the invention encompasses the products of the screening process whether alone, in the form of a pharmaceutically acceptable salt, in combination with one or more other active compounds and/or in combination with one or more pharmaceutically acceptable carriers. Processes for making a pharmaceutical composition are also provided in which a chemical compound identified by the process of the invention is brought into association with a pharmaceutically acceptable carrier.

The chemical compound may be an organic chemical and may contain amino acids or amino acid analogues. Preferably however the chemical compound is a polypeptide or a polypeptide which has been chemically modified to alter its specific properties, such as the affinity of binding to the CD81 protein or functional equivalent thereofor its stability *in vivo*.

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According to a further aspect of the invention, there is provided a nucleic acid encoding CD81 protein or functional equivalent thereof for use in diagnosis or therapy of HCV.

The nucleic acid may encode any of the CD81 protein, or functional equivalent thereof. Changes may be made at the nucleotide level by addition, substitution, deletion or insertion of one or more nucleotides, which changes may or may not be reflected at the amino acid level, dependent on the degeneracy of the genetic code.

The nucleic acid may be included in a vector, optionally an expression vector permitting expression of the nucleic acid in a suitable host to produce CD81 protein or functional equivalent thereof.

The identification of the DNA encoding the HCV receptor, namely CD81, makes available the full power of molecular biology for the molecular analysis of HCV and in particular its infectious mechanism, offering for the first time the possibility of designing methods of treating the virus. PCR methods may be used to identify cells carrying the receptor and DNA molecules may be designed to act as polymerase chain reaction (PCR) primers in this connection. Although CD81 is widespread and is associated with normal human function, the present invention includes antisense molecules inhibiting CD81 production for use in the treatment of HCV and in the manufacture of a medicament for the treatment of HCV infection.

According to a further aspect of the invention, there is provided an antibody to CD81 protein or functional equivalent thereof for use in the treatment of an HCV infection and in the manufacture of a medicament for the treatment of an HCV infection. The antibody is preferably a monoclonal antibody. Such an antibody can be used to temporarily block the CD81 receptor preventing infection from HCV, for example, immediately after an accidental infection with HCV-infected blood.

At present, the only available animal model of HCV infection is the chimpanzee, which is a protected species. Experiments on such animals pose a number of difficulties which together result in a very considerable expense (a one year experiment with one chimpanzee can cost $70,000). Compared to this, a mouse model would be far more acceptable.

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Unfortunately, as described below the HCV receptor, whilst ubiquitous in humans and found in chimpanzees, is absent in other mammals. A transgenic mammal, for example a mouse, carrying the HCV receptor on the cell surface would be of great benefit to HCV research and the development of vaccines.

According to a further aspect of the invention, there is provided a transgenic non-human animal, suitably a mammal such as a mouse, carrying a transgene encoding a CD81 protein or functional equivalent thereof.

The transgenic animal of the invention may carry one or more other transgenes to assist in maintaining an HCV infection.

There is also provided a process for producing a transgenic animal comprising the step of introducing a DNA encoding a CD81 protein or functional equivalent thereofinto the embryo of a non-human mammal, preferably a mouse.

Preferably the CD81 protein or functional equivalent thereof is a human CD81.

Brief Description of the Drawings

Figure 1A is a schematic description of primary, secondary and tertiary rounds of screening.

Figure 1B is a schematic description of the final round of screening.

Figure 2 is a FACS scan analysis of E2 bound cells.

Detailed Description of the Invention

Example 1. Recombinant E2, cell lines, vector DNA, and antibodies used in the present study.

The recombinant E2 used in this screening was produced in CHO cells (E2-CHO) (WO

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97/09349). E2-CHO binds to the human T cell lymphoma cell line Molt-4. A subline of Molt-4 (A2A6), which binds more E2-CHO molecule on its surface than its parental line was chosen for the source of RNA, expecting that this subline may have a higher representation of the transcript encoding the E2 binding molecule. WOP is a NIH3T3-derived cell which expresses polyoma T antigen. In this cell line, plasmids containing the polyoma replication origin can be amplified episomally. This allows the recovery of recombinant DNA constructed with pCDM8 (Invitrogen) from selected transfectants, which contains the polyoma replication origin and is designed for the manipulation of expression libraries in eukaryotic cells.

A mouse monoclonal anti E2 antibody (291A2) was used for detection of E2-CHO bound on the cell surface of transfectants.

Example 2: Construction of cDNA library

Total RNA was extracted from the A2A6 cell line according to the method described by Chomczinsky and Sacchi (Chomczinsky, P. And Sacchi, N. *Anal Biochem.162:* 156-159, 1987.). Poly(A)+ was enriched twice using oligo(dT) cellulose. Starting from 2µg of this RNA as a template, the double strand complementary DNA was synthesized using a Superscript II cDNA synthesis kit (Life Technologies) in the presense of oligo(dT) (100ng) and random hexamer primers (100ng). The cDNA was blunt-ended with T4 DNA polymerase, and was ligated with a *Bst*XI linker, which allows the insertion of the fragment into the same restriction site in the polylinker region of the expression vector pCDM8. The linker-ligated cDNA was phenol-extracted and ethanol precipitated using ammonium sulphate to remove free mononucleotides, followed by Sephacryl 500 chromatography (Lifetechnologies) to size-fractionate the cDNA. The purified cDNA fragment over 500bp were pooled and ligated with *Bst*XI - digested pCDM8 at a molecular ratio of approximately 1:1. This final ligation reaction was used from transformation of *E. Col* MC1061/P3 by electroporationusing Gene-Pulser (BIORAD). A total of $2x10^6$ cfu was amplified and pooled in liquid bacterial culture as a cDNA library.

Example 3: Library screening

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The screening procedure was based largely on the method described by Campbell *et al.* (Campbell, I. G., Jones, T.A., Foulkes, W. D. And Trowsdale, J. Cancer Res. 51: 5329-5338, 1991). Enrichment was carried out using magnetic beads (the first to the third round) (Figure 1A) and panning techniques (the fourth round). (Figure 1B).

3.1 The first round of screening

A total of 375μg of amplified DNA, which represents $2x10^6$ of independent cDNA clones, was prepared. In each transfection, 25μg of DNA was mixed with 10^7 WOP cells using the Gene-Pulser electroporator (BIORAD) under the condition of 300V/500μF. Fifteen sets of transfections were performed. After transfection, cells were incubated at 37°C for 2 days and then the cells were detached by trypsinization and washed with PBS supplemented with 5% FCS and 0.5mM EDTA twice by centrifugation at 360g for 10min at 4°C. The cell pellet was resuspended in PBS supplemented with 5% FCS and 0.5mM EDTA (10^7 cells/ml) and then E2-CHO was added to the cell suspension at a concentration of 10μg/ml. The cells were incubated on ice for 60 min. After washing twice with PBS supplemented with 5% FCS and 0.5mM EDTA, the cell suspension was incubated with 291A2 antibody on ice for 30 min. After washing twice with PBS supplemented with 5% FCS and 0.5mM EDTA, 10μl of Dynabeads (DYNAL) coupled with goat anti-mouse IG was added to the cell suspension. The mixture was gently agitated using a Coulter Mixer (Coulter) for 60 min at 4°C. Bound cells were separated using Magnetic Particle Concentrator (DYNAL) from non-binders, according to the manufacturer's instructions, thus enriching E2-binding transfectants. Plasmid DNA was recovered from the bound transfected cells using the protocol described by Campbell *et al.* (Campbell, I. G., Jones, T.A., Foulkes, W. D. And Trowsdale, J. Cancer Res. 51: 5329-5338, 1991). *E. coli* MC1061/P3 was transformed with this plasmid by electroporation. This DNA pool is referred to as the first enriched pool (1°EP).

3.2 The second round of screening

A total of 150μg of amplified DNA derived from 1°EP was prepared and 6 sets of the transfection were performed and transfectants were enriched using the same condition as in the first screening. This DNA pool is referred to as 2°EP.

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3.3 The third round of screening

A total of 25μg of amplified DNA derived from 2°EP was prepared and one set of the transfection was performed. Transfectants were enriched using the same condition as in the first screening. During this separation step, transfectants formed aggregates, which might be caused by expression of irrelevant adhesion molecules. This could decrease the efficiency of enrichment because these aggregates contained magnetic beads nonspecifically. To circumvent this potential problem, transfectants after the second separation by Magnetic Particle Concentrator were diluted and plated on Terasaki plates. Approximately 100 of single cells identified under microscope were pooled and plasmid DNA was extracted from them. The DNA pool prepared from this step is referred to as 3°EP.

3.4 The fourth round of screening

291A1 monoclonal antibody was incubated in a petri dish (90mm) at a concentration of 10μg/ml overnight at 4°C.

A total of 25μg of amplified DNA derived from 3°EP was prepared and one set of the transfection was performed. The transfected cells were incubated with E2-CHO as described above, and placed onto the 291A2-coated plates for 60 min at 4°C. After rinsing with a large excess of PBS supplemented with 5% FCS and 0.5mM EDTA twice, the bound cells were directly treated with the lysing solution and plasmids were extracted as described as before. This DNA pool is referred to as 4°EP.

3.4 Identification of cDNA encoding a molecule binding to the recombinant E2

DNA was isolated form single colonies derived from 4°EP. A single transfection was performed for each plasmid preparation using the same conditions as used for the previous screening steps. E2-binding of the transformants was detected using a phycoerythrin-conjugated monoclonal Fab fragment of goat anti-mouse Ig instead of the antibody-coupled Dynabeads. Transfectants of 3°EP and 4°EP were also analyzed in the same way. The E2-bound cells were detected on FACScan (Becton Dickinson) and analyzed with

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LYSIS II program (Becton Dickinson) (Figure 2). E2-CHO binds increasingly as the purification step advances. A single clone P3 showed strong E2-binding.

Example 4: DNA sequencing determination and analysis.

P3 contains a insert of approximately 1 kb. The DNA sequence of the insert of the cDNA clone which confers E2-binding to WOP upon transfection was determined by an automated sequencing system using the T7 primer, whose sequence is located adjacent the cloning site of pCDM8. The sequence was screened through the GenBank databases using the GCG programs on a UNIX computer. This analysis revealed that the 5' part of P3 insert is identical to human CD81 (TAPA-1). Restriction analysis of P3 using three enzymes (*Bst*XI), *Hinc*II and *Nco*I) also agreed with the restriction map of human CD81 cDNA.

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Claims

1 A CD81 protein, or a functional equivalent thereof for use in the therapy or diagnosis of HCV.

2 A method for treating an infection of HCV comprising administering to a patient a therapeutically effective amount of a CD81 protein, or a functional equivalent thereof to reduce the infectivity of the virus.

3 A pharmaceutical composition comprising a CD81 protein, or a functional equivalent thereof, optionally as a pharmaceutically acceptable salt, in combination with a pharmaceutically acceptable carrier.

4 A process for preparing a pharmaceutical composition, in which a CD81 protein, or a functional equivalent thereof is brought into association with a pharmaceutically acceptable carrier.

5 Use of a CD81 protein, or a functional equivalent thereof in the manufacture of a medicament for the treatment of an HCV infection.

6 An assay for HCV antibodies in a serum sample comprising the step of allowing competitive binding between antibodies in the sample and a known amount of CD81 protein, or a functional equivalent thereof and measuring the amount of the known HCV protein bound.

7 A diagnostic kit comprising a CD81 protein, or a functional equivalent thereof optionally labelled.

8 A method for screening chemical compounds for ability to bind to the region of HCV responsible for binding to a host cell, comprising measuring the binding of a chemical compound to be screened to a CD81 protein, or a functional equivalent thereof.

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9 A transgenic non-human mammal, carrying a transgene encoding CD81 protein, or a functional equivalent thereof.

10 A process for producing a transgenic animal comprising the step of introducing a DNA encoding a into the embryo of a non-human mammal, preferably a mouse.

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Abstract

Hepatitis Receptor Protein and DNA

The use of CD81 protein and polynucleic acid in the therapy and diagnosis of hepatitis C and pharmaceutical compositions, animal models and diagnostic kits for such purposes.

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Figure 1A

1°, 2°, 3° round screening

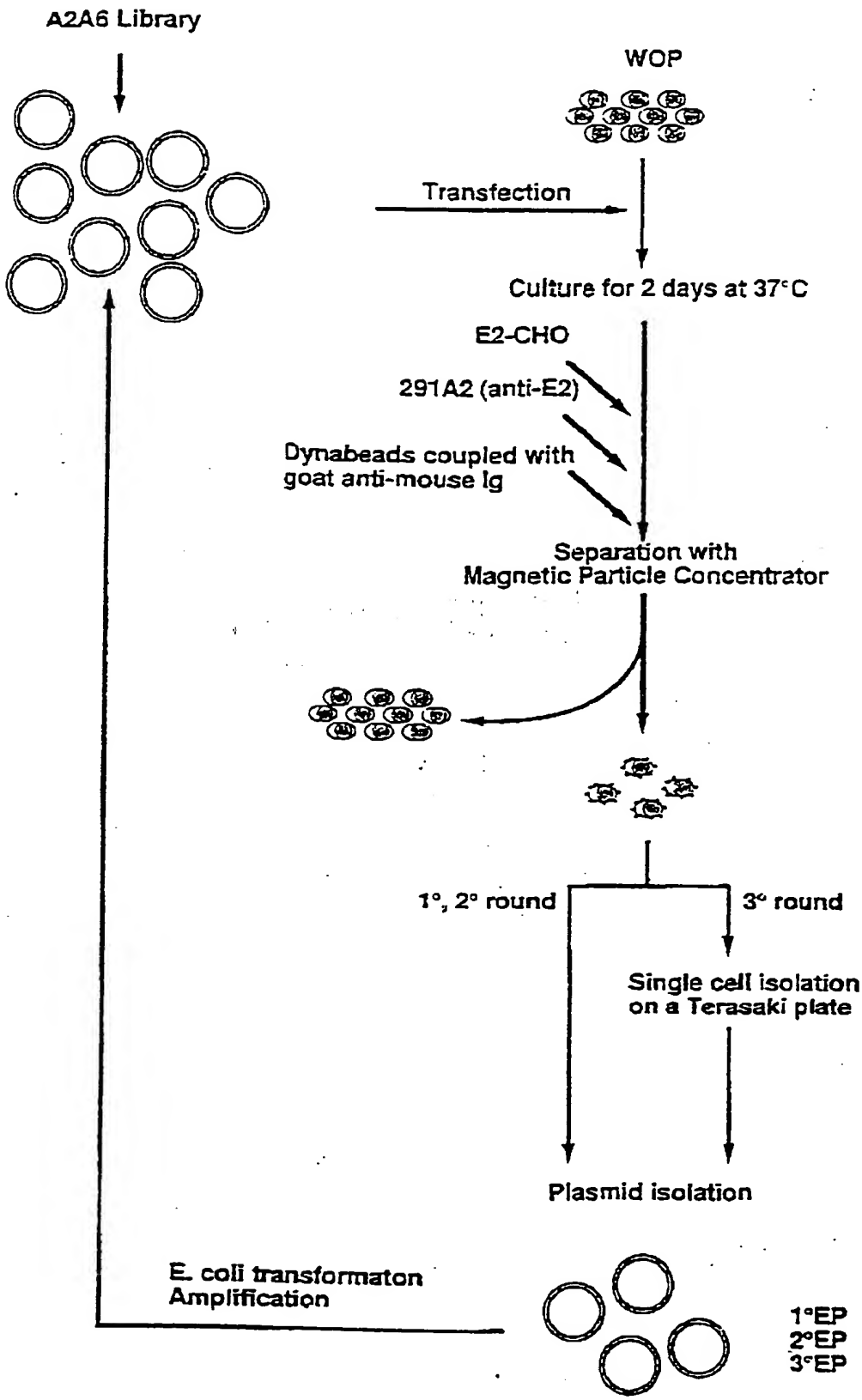

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Figure 1B

4° round screening

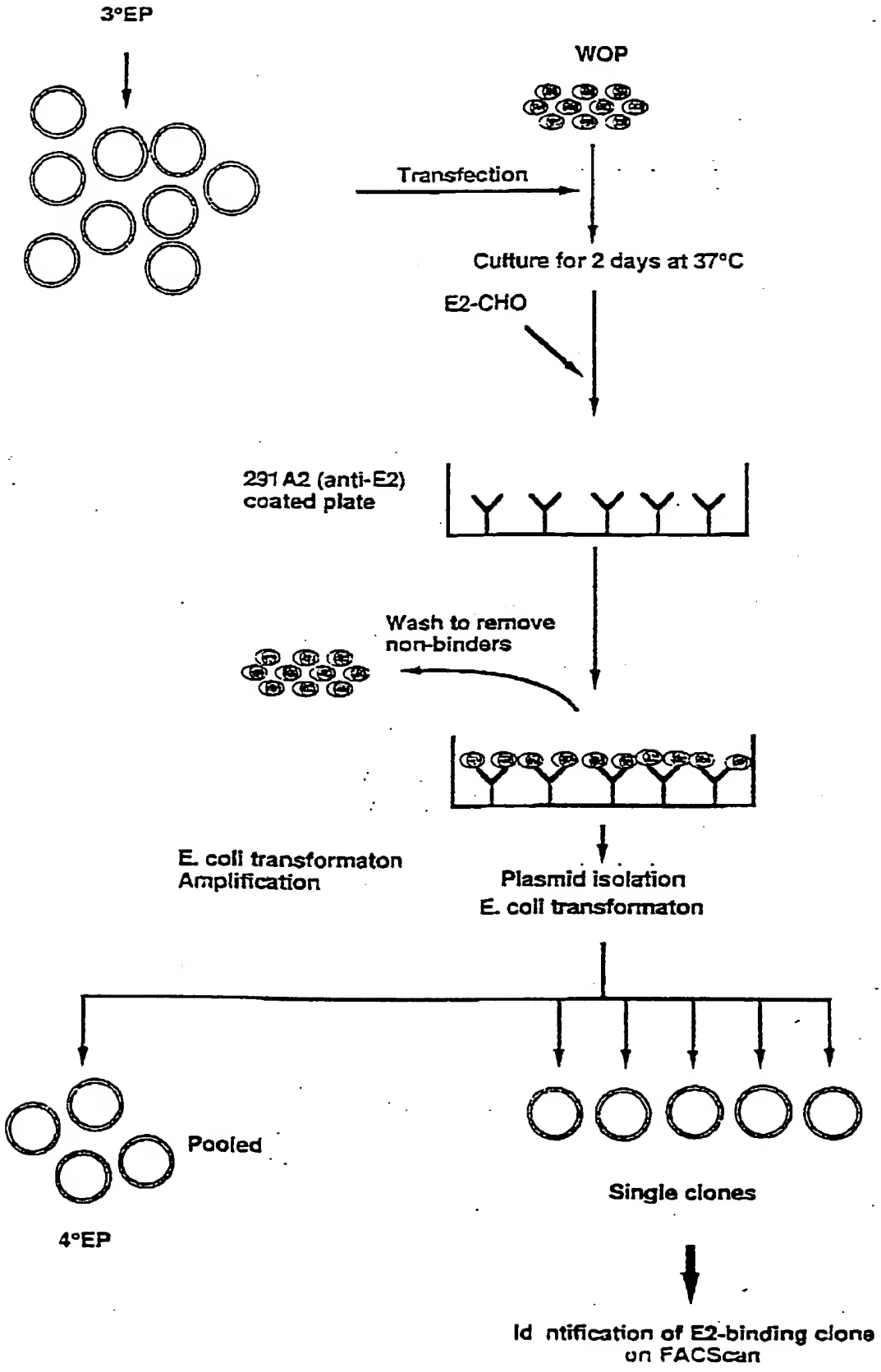

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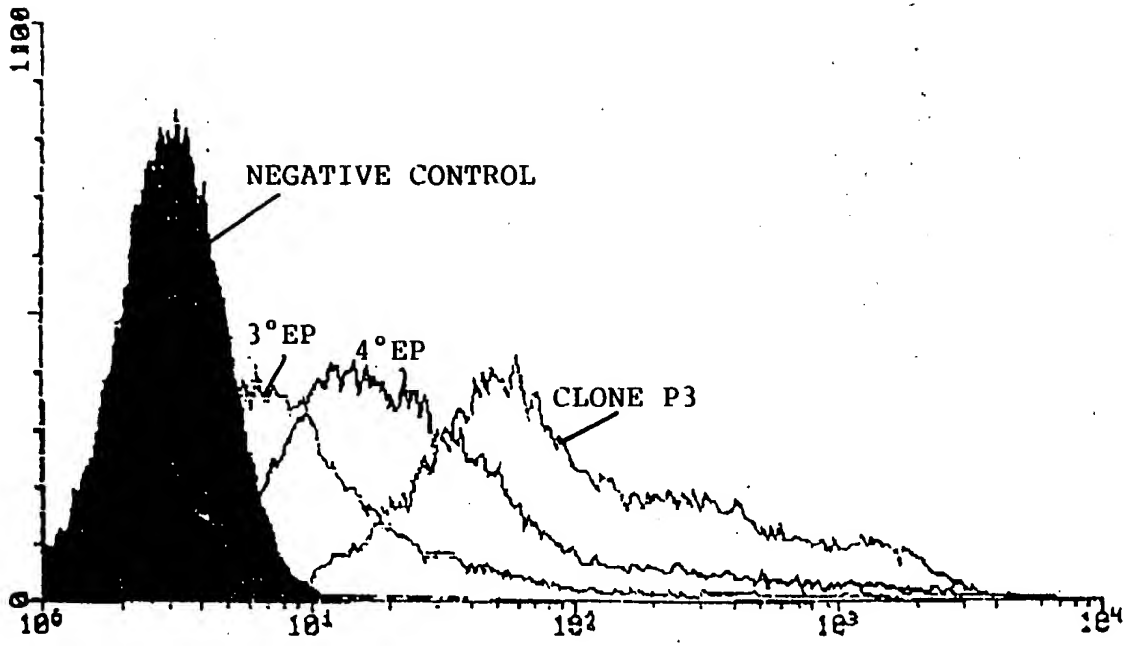
FIGURE 2

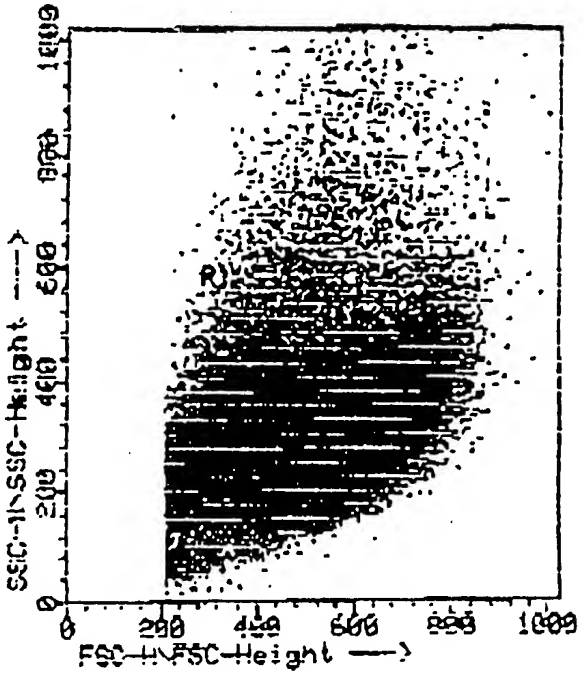
BECTON
DICKINSON

LYSYS II Ver 1.1 2/6/92

DATE: 26-SEP-97 TIME: 16:27:22

SELECTED PREFERENCES: Arithmetic/Linear





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